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Comparison of the serotonergic nervous system among Tunicata: implications for its evolution within Chordata

Thomas Stach

Royal Swedish Academy of Sciences, Kristineberg's Marine Researchstation, 45034 Kristineberg, Sweden

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Abstract

Using immunohistochemistry in combination with confocal laser scanning microscopy, the serotonergic nervous systems of major tunicate taxa were studied in three-dimensional detail. Organisms analyzed included aplousobranchiate, phlebobranchiate, and stolidobranchiate ascidian larvae, appendicularian juveniles and adults, and doliolid oozooids. Outgroup comparisons to notochordates showed that the serotonergic nervous system of the last common ancestor of Chordata consisted of two elements: (i) an anterior concentration of serotonergic cell bodies, and (ii) a fiber network that followed posteriorly and gave rise to fiber tracts that descended towards the effective somatic lateral musculature. Within Tunicata, the nervous systems of Appendicularia and Aplousobranchiata appear serotonin-reduced or negative. This could be interpreted as a heterochronic reduction and a synapomorphy between Appendicularia and Aplousobranchiata. In this hypothesis, free-living Appendicularia are derived within Tunicata, and a biphasic life cycle with a free-swimming larva and a sessile, ascidian-like adult is most parsimoniously reconstructed for the last common ancestor of Tunicata. The close spatial relation of the serotonergic cell cluster with the statocyte complex suggests a function as an integrative control center for the coordination of locomotion. A similar anterior concentration of serotonergic nerve cells is known from tornaria larvae.

Keywords: Chordate; Urochordate; Tunicate; Phylogeny; Serotonin; 5-HT

See also **Electronic Supplement** at: http://www.senckenberg.de/odes/05-02.htm

Introduction

Structures of the nervous system can preserve phylogenetically informative characters even in otherwise disparate taxa (e.g. Hanström 1928; Hessling and Westheide 2002; for Chordata see reviews by Nielsen 1999; Meinertzhagen and Okamura 2001; Nieuwenhuys 2002). Serotonin is an ancient neurotransmitter present in most bilaterian taxa (see; e.g., Hay-Schmidt 2000). To date, the organization of the serotonergic nervous system in Chordata is known for Petromyzontida (e.g. van Dongen et al. 1985; Hay-Schmidt 2000), Gnathostomata (e.g. Ekström et al. 1985; van Mier et al. 1986), and, to some extent, Cephalochordata (Holland and Holland 1993; Candiani et al. 2001; Morikawa et al. 2001). For the craniate taxa functional data on the serotonergic part of the nervous system are also available (van Mier et al. 1986; Grillner and Matsushima 1991). However, for the third major taxon of the Chordata, the Tunicata, the serotonergic nervous system remains hardly known. Pennati et al. (2001) demonstrated the presence of serotonin in the larval brain and also the tail of *Phallusia*

E-mail address: thomas.stach@kmf.gu.se (T. Stach).

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mammillata, a phlebobranch ascidian species. Previous attempts to study the distribution of serotonin using immunofluorescence in adult nervous systems of several ascidian species have returned largely negative results (Fritsch et al. 1982; Georges 1985).

Tunicates display an enormous diversity of life cycles, and recent molecular studies led to two conflicting hypotheses concerning the phylogenetic position of Appendicularia. Wada (1998), Swalla et al. (2000), and Winchell et al. (2002) supported the hypothesis that Appendicularia were the sister group to the remaining tunicates, and thus suggested that the last common ancestor of Tunicata was free-living. Stach and Turbeville (2002, in press) included Aplousobranchiata in their analyses and found that Appendicularia were the sister group to aplousobranchiate ascidians. Besides the analysis of 18S rDNA sequences these authors pointed out that aplousobranchiate ascidians and appendicularians shared a horizontally oriented tail in their early developmental stages. However, this character is also found in Perophoridae, a taxon that is nested within the phlebobranchiate ascidians. Thus there are currently no undisputed morphological characters known that support either of the two hypotheses.

Tunicata is one of three major taxa of Chordata. Chordata, Echinodermata, Hemichordata, and possibly *Xenoturbella* sp. (Bourlat et al. 2003), constitute the Deuterostomia. Knowledge of the last common ancestor of Tunicata influences our perception of the last common ancestor of Chordata, which in turn is important to understand the evolution of Deuterostomia. A major controversy in the evolution of Deuterostomia concerns the phylogenetic position of Hemichordata. Molecular studies strongly support a sister group relationship between Hemichordata and Echinodermata (recently reviewed in Jenner and Schram 1999; Winchell et al. 2002). Morphological studies often find arguments to support a sister group relationship between Enteropneusta and Chordata (e.g. Ax 2001; Nielsen 2001; Lowe et al. 2003; Nezlin and Yushin 2004), though not all morphological studies agree.

The potentially conservative aspect of the nervous system, the ubiquitous distribution of the serotonin neurotransmitter, and the scarcity of data on the serotonergic nervous system in tunicates, make this system a promising candidate to contribute to phylogenetic discussions. Here, I present a comparative study of the serotonergic part of the central nervous system for phlebobranch, stolidobranch, and aplousobranch ascidians, as well as for appendicularians and doliolids. The results allow the reconstruction of details of the serotonergic nervous system for the last common ancestor of Tunicata and Chordata, show some similarities to the serotonergic nervous system of tornaria larvae, and in addition suggest an integral function in chordate locomotion.

Colour versions of Figs. 1–4 and 6, as well as additional illustrations are available from an Organisms Diversity and Evolution Electronic Supplement (www.senckenberg.de/odes/05-02.htm).

Material and methods

Specimens

Table 1 lists the species examined in the present study along with information on the respective number and stages of animals analyzed.

Origin of animals, fixation, storage

Solitary ascidians: Gametes of two individuals were dissected and mixed on a 220 µm mesh gauze on top of a beaker containing filtered seawater (pore size 0.4 µm). Thus, fertilized eggs sank to the bottom and superfluous sperm in the water column was decanted three times. Development of animals was followed under a dissecting microscope. Timed stages were fixed in 1.5% paraformaldehyde in seawater for at least 2 h to a maximum fixation time of 12 h. If animals were stored, they were washed in distilled water for 10 min, transferred to 100% methanol, and kept in a refrigerator at 4 °C. All animals were used within 6 weeks after fixation. Larvae of colonial ascidian species were dissected from the atria or brooding pouches, respectively, and processed in the same way. Planktonic species were caught with a 15-min drift tow at about 5 m depth. Mesh size of the plankton net was 220 µm. All animals were immunolabeled against serotonin for microscopic examination.

Immunohistochemistry

If necessary, animals were rehydrated in distilled water for 10 min. The larval tunic of ascidian species proved to be a major obstacle against antibody penetration. Various methods – e.g., acetone treatment, microwaving, sonication, freeze-and-thaw cycles, protease digestion – were evaluated as to their effectiveness in enhancing antibody penetration. The most successful procedure was as follows. Larvae were incubated for 5 min in 5% cellulase (Sigma, St. Louis, Missouri, USA) in phosphate-buffered saline (PBS, 0.15 M, pH 5.5) at 37 °C. After subsequent heating to 72 °C, larvae were cooled down on ice and washed with PBS (0.15 M, pH 7.4) 3 times for 10 min each. Prior to incubation with the primary antibody, all specimens were treated for 1 h at room temperature in PBS (0.15 M, pH 7.4) containing 0.1% Triton-X-100, 2% Bovine Serum Albumine (BSA), and 0.05% NaN_3. Incubation in primary antibody anti-serotonin (Sigma, St. Louis, Missouri, USA)

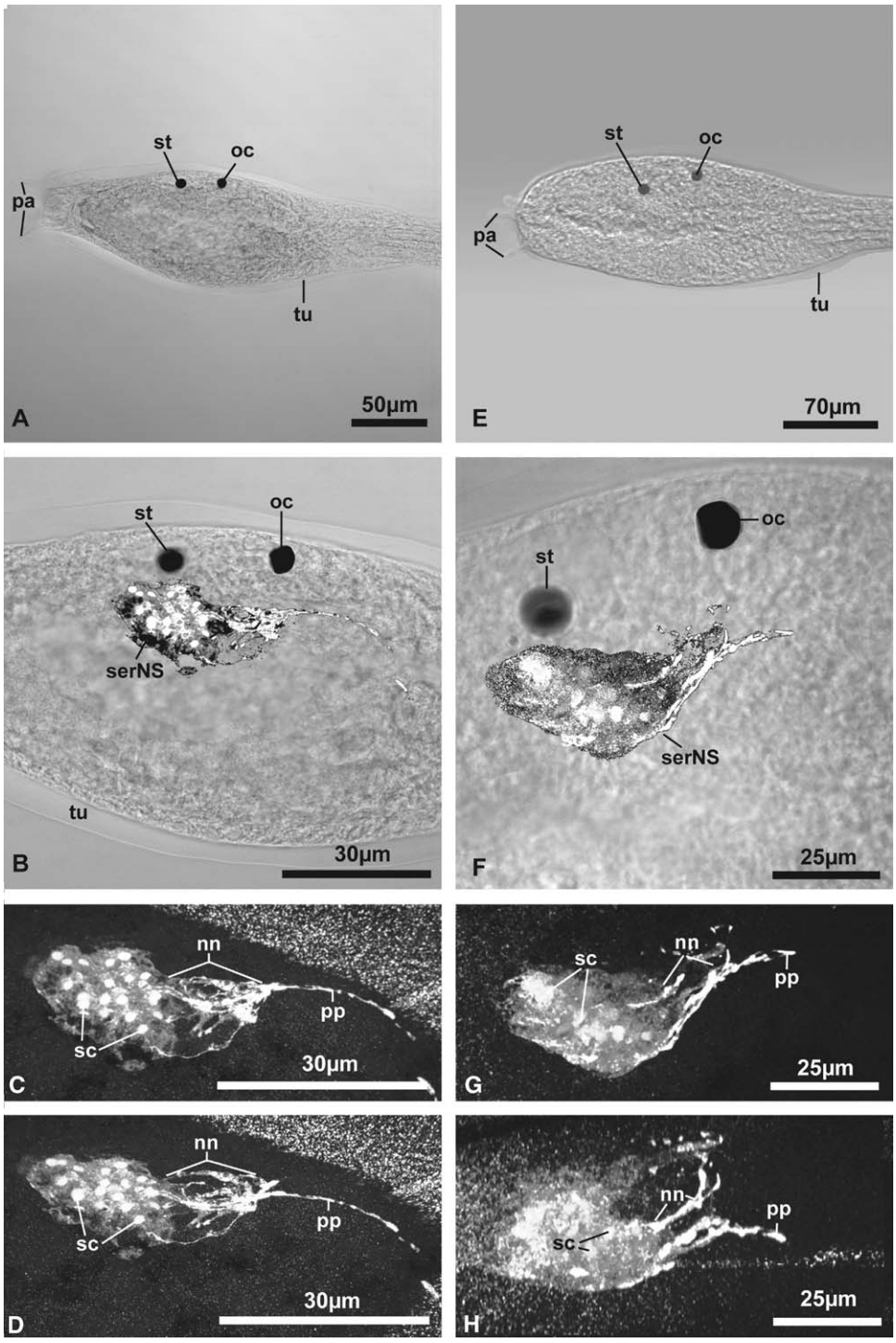

Fig. 1. (A–D). *Herdmania momus* (Pyuridae, Stolidobranchiata). Larval stage 21.5 h post-fertilization, approx. 20 °C. (A) General light-microscopic aspect. (B) Superimposed images of transmitting light-microscopic image with projection of serotonergic nervous system; serotonergic nervous system: background-removed part of a projection of 104 confocal optical sections taken at 0.5 µm intervals. (C) Details of the serotonergic nervous system showing the entire projection of optical sections used in (B). (D) Serotonergic nervous system, oblique ventral aspect; projection as in (B,C) but rotated by 45°. (E–H) *Ascidia interrupta* (Ascidiidae, Phlebobranchiata). Larval stage 21.5 h post-fertilization, approx. 20 °C. (E) General light-microscopic aspect. (F) Superimposed images of transmitting light-microscopic image with projection of serotonergic nervous system; serotonergic nervous system: background-removed part of a projection of 93 confocal optical sections taken at 0.5 µm intervals. (G) Details of the serotonergic nervous system showing the entire projection of optical sections used in (F). (H) Dorsal aspect; projection as in F, G, but rotated by 90°. Abbreviations: nn = fiber net, oc = ocellus, pa = papilla, pp = posterior projection, serNS = serotonergic nervous system, sc = serotonergic cell, st = statocyte complex, tu = tunic.

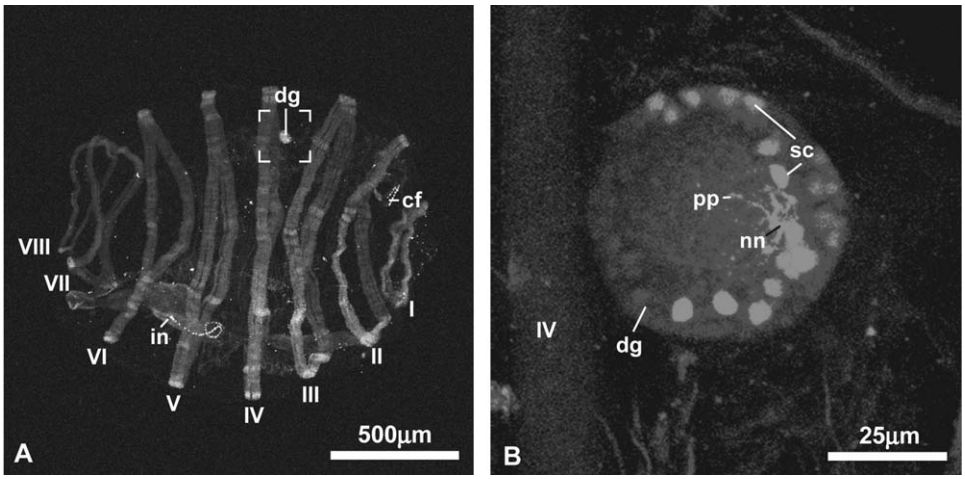

Fig. 2. Oozooid of *Doliolum nationalis* labeled with antibodies against serotonin; projections of confocal optical sections. (A) Overview, anterior to the right, dorsal to the top; 97 optical sections taken at 5 µm intervals; [ ] = area enlarged in (B). (B) Dorsal ganglion; 67 optical sections taken at 1.5 µm intervals. Abbreviations: cf = ciliated funnel, dg = dorsal ganglion, in = intestine, nn = fiber net, pp = posterior projection, sc = serotonergic cell, I–VIII = muscle bands.

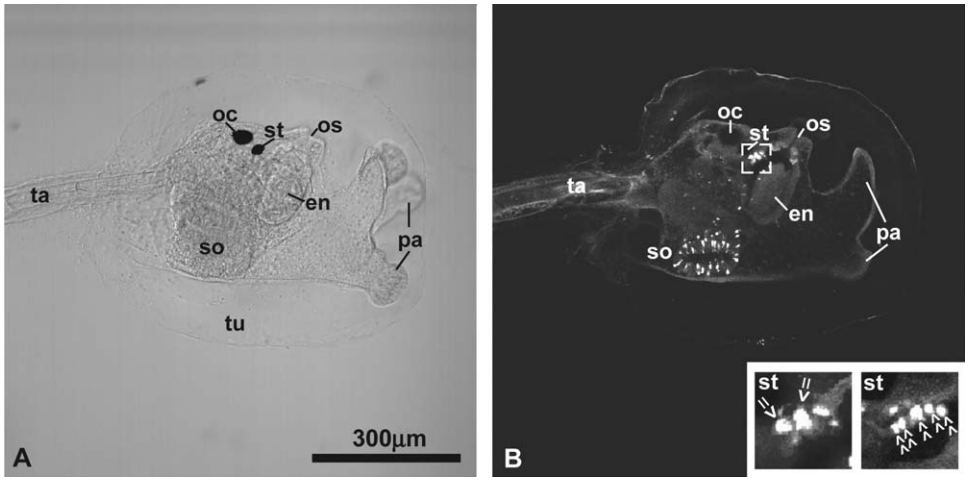

Fig. 3. *Clavelina oblonga* larva released from atrium. (A) General light-microscopic aspect. (B) Confocal optical section of same larva shown in (A) labeled with antibodies against serotonin; no projection shown, because outer tunic was labeled at various positions, thereby masking internal signal; representative section from a series of 57 confocal optical sections taken at 1 µm intervals. Insets: area indicated by [ ] enlarged, two confocal optical sections taken 7 µm apart; arrows point to immunopositive cells lining the sensory vesicle; double arrowheads point to immunopositive cells in the pharyngeal epithelium. Abbreviations: en = endostyle, oc = ocellus, os = oral siphon, pa = papilla, so = stomach, st = statocyte complex, ta = tail, tu = tunic.

was carried out in preincubation fluid at a dilution of approximately 1:100 for 12–24 h in a humid chamber at room temperature. Animals were washed six times for 10 min in PBS (0.15 M, pH 7.4). Incubation in secondary antibody Alexa Fluor® 546 goat anti-rabbit (Molecular Probes, Eugene, Oregon, USA) was carried out in preincubation fluid without BSA at a dilution of 1:200 for 6–24 h in a humid chamber at room temperature. Specimens were washed 12 times for 10 min, transferred to distilled water, and dehydrated through an ethanol series. Animals were mounted on polylysine (Sigma, St. Louis, Missouri, USA) covered microscope slides and cleared with Murray's fluid (2 parts benzyl benzoate:1 part benzyl alcohol). Two different controls were processed the same way, one with primary antibodies omitted, the second with secondary antibodies omitted.

Microscopy

All specimens were studied with a Nikon Eclipse E800 microscope equipped with a Radiance2000 confocal laser scanning system (BioRad). Stacks of confocal optical sections were recorded, applying appropriate

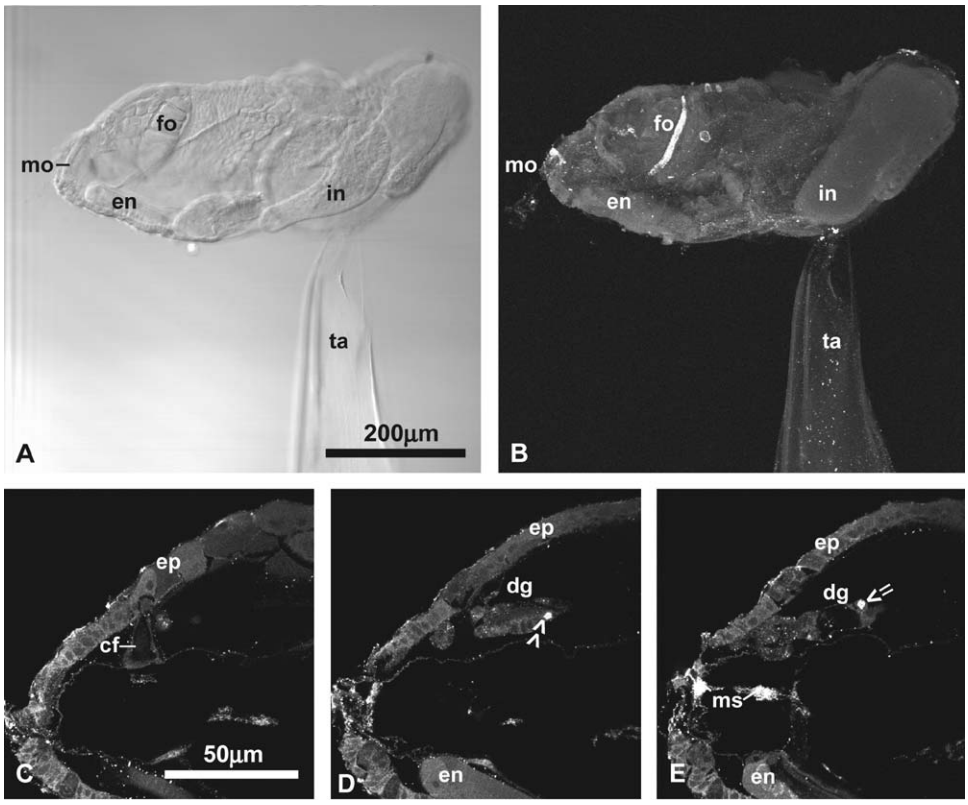

Fig. 4. *Oikopleura fusiformis* (Appendicularia) labeled with antibodies against serotonin. (A) Light-microscopic aspect. (B) Projection of 71 confocal optical sections taken at 2 µm intervals. (C–E) Three confocal optical sections taken at 20 µm intervals; double arrowhead and arrow mark cells labeled in the dorsal ganglion. Abbreviations: cf = ciliated funnel, dg = dorsal ganglion, en = endostyle, ep = epidermis, fo =Fol's oikoplast, in = intestine, mo = mouth, ms = mucus strand, ta = tail.

Table 1. Information on specimens examined

Species	Family	Order	# specimens examined	Ontogenetic stage	Serotonin in CNS[a]
Oikopleura rufescens Fol, 1872	Oikopleuridae	Appendicularia	4	Adult	Not detected
Oikopleura fusiformis Fol, 1872	Oikopleuridae	Appendicularia	3	Juvenile/adult	Few cells[b]
Fritillaria sp. (Quoy & Gaimard 1833)	Fritillariidae	Appendicularia	1	Adult	Not detected
Clavelina oblonga Herdmann, 1880	Clavelinidae	Aplousobranchiata	23	Larva	Few cells[c]
Eudistoma olivaceum (Van Name, 1902)	Clavelinidae	Aplousobranchiata	7	Larva	Not detected
Aplidium constellatum (Verrill, 1871)	Polyclinidae	Aplousobranchiata	13	Larva	Not detected
Didemnum cf. *candidum* Savigny, 1816	Didemnidae	Aplousobranchiata	4	Larva	Not detected
Ascidia interrupta Heller, 1878	Ascidiidae	Phlebobranchiata	11	Larva	Distinctly present[d]
Ciona intestinalis Linné, 1767	Cionidae	Phlebobranchiata	9	Larva	Present[e]
Herdmania momus (Savigny, 1816)	Pyuridae	Stolidobranchiata	18	Larva	Distinctly present
Microcosmus exasperatus Heller, 1878	Pyuridae	Stolidobranchiata	5	Larva	Present
Styela plicata Leseur, 1823	Styelidae	Stolidobranchiata	7	Larva	Distinctly present
Molgula occidentalis Traustedt, 1883	Molgulidae	Stolidobranchiata	4	Larva	Present
Doliolum nationalis Borgert, 1894	Doliolidae	Thaliacea	4	Oozooid	Distinctly present
Thalia democratica (Forskål, 1775)	Salpidae	Thaliacea	3	Oozooid	Present

[a]CNS = central nervous system.
[b]Few cells (*Oikopleura fusiformis*) = two cells in dorsal ganglion.
[c]Few cells (*Clavelina oblonga*) = two cells next to sensory vesicles plus five cells in pharyngeal epithelium.
[d]Distinctly present = anterior cell aggregation, posterior fiber net, posterior projection.
[e]Present = signal of several cells detected, morphology unclear; see text for details.

filter settings. A transmitting light microscopic picture of the same frame and magnification was recorded immediately after collection of the stacks of confocal optical sections. BioRad's LaserSharp2000 and Confocal Assistant software was used to analyze the images.

Results

Stolidobranchiata

Fig. 1A–D shows light-microscopic images of a larva of *Herdmania momus* after hatching (21.5 h post-fertilization, at 20 °C). The part of the nervous system in the larval trunk that is immunopositive to labeling with antibodies against serotonin in *H. momus* is seen in Fig. 1B–D.

The serotonergic nervous system consists of two parts. Anteriorly, about 20 cells form a cluster of approximately 20 μm length, 15 μm height, and 15 μm depth. Perikarya stain intensely and are of a spherical shape with a diameter of approx. 2 μm (Fig. 1C, D). Cell counts in this anterior region of different specimens varied between 17 and 20 ($n = 4$). The discrepancy might be related to differences in staining intensity. The anterior cell bodies are closely packed and seem to possess short fibers establishing contact with one another. Note the close spatial relationship of this cluster of serotonergic perikarya to the larval statocyte complex that becomes obvious when the fluorescent image is projected onto the same light-microscopic frame (Fig. 1B).

Several of the serotonergic neurons have fibers projecting posteriorly. These fibers form a conspicuous network immediately posterior to the cluster of perikarya, and neurons seem to form lateral connections in it (Fig. 1C, D). The entire network is of conical shape; it is wide anteriorly and narrows at its posterior end, where one single longer nerve fiber emerges. This fiber projects in a slightly ventrally bent curve for another 30 μm further posteriorly.

Labeling with antibodies against serotonin in the larvae of *Microcosmus exasperatus* (Pyuridae), *Styela plicata* (Styelidae), and *Molgula occidentalis* (Molgulidae), was detected close to the cerebral vesicle. However, only *S. plicata* showed sufficient preservation to ascertain the presence of an anterior concentration of cells followed by a posterior fiber net and a posteriorly projecting fiber.

Phlebobranchiata

Fig. 1E–H shows light-microscopic images of a larva of *Ascidia interrupta* after hatching (21.5 h post-fertilization, 20 °C). The part of the nervous system in the larval trunk that is immunopositive to labeling with antibodies against serotonin in *A. interrupta* is depicted in Fig. 1F–H. Again, the serotonergic nervous system consists of two parts. The anterior perikarya form a cluster of approximately 40 μm length, 22 μm height, and 20 μm depth. Individual cells are harder to discern in this species, because spheres of the appropriate size range (around 2 μm) display various intensities of antibody labeling. Estimates of the number of cells in the anterior region in different specimens varied between 36 and 41 ($n = 3$). The anterior cell bodies are closely packed. Note the close spatial relationship of this cluster of serotonergic cells to the larval statocyte complex (Fig. 1F).

Fibers projecting posteriorly from the serotonergic cells form a less extensive network compared to *H. momus* larvae. In this network several fibers run almost parallel to one another for about 15 μm (Fig. 1G, H), although some lateral connections seem to be present. One single longer fiber projects in a slightly ventrally bent curve for another 12 μm further posteriorly (Fig. 1F).

Nervous structures in the larvae of *Ciona intestinalis* stained positively with antibodies against serotonin; fixation quality was too poor to record the detailed morphology.

Doliolidae

Labeling with antibodies against serotonin in the oozooid of *Doliolum nationalis* is seen in Fig. 2. Immunoreactivity is confined to three areas: the dorsal ganglion, the ciliated funnel, and the intestinal tract.

In the dorsal ganglion approximately 20 serotonergic cells are distributed in a u-shaped pattern (Fig. 2B). There are 4–5 positively labeled cells laterally on each side. About 5 cells with fainter staining are situated at the anterior border of the ganglion. Immediately behind this group of anterior cells lie another 5 cells that stain intensely with antibodies against serotonin. From these latter cells short fibers emerge that form a network from which a slightly longer fiber projects posteriorly.

Preliminary investigation demonstrates that several cells but no fibers were labeled with antibodies against serotonin in the oozooid of *Thalia democratica*.

Aplousobranchiata

Fig. 3 shows light microscopic images of a larva of *Clavelina oblonga*. Immunoreactivity for antibodies against serotonin is seen in Fig. 3B. There are numerous serotonergic cells labeled in the stomach wall. Inset in Fig. 3B shows immunoreactive cells close to the larval statocyte complex. Anterior to the statocyte complex on its ventral side are cell bodies of two cells that show

intense labeling (arrows in Fig. 3B, left inset). One of these cells bears an apical process that projects dorsally and in the direction of the statocyte complex. Next to these two serotonergic cells is a row of 5 cells that react with the antibodies against serotonin (double arrowheads in Fig. 3B, right inset). These cells are situated in the dorsal epithelium of the branchial basket. This is the site where in the adults the ciliated funnel that connects the neural gland to the pharynx opens. No labeling with antibodies against serotonin was detected in earlier ontogenetic stages of *C. oblonga* (Clavelinidae).

Staining experiments with antibodies against serotonin in the larvae of *Eudistoma olivaceum* (Clavelinidae), the larvae of *Aplidium stellatum* (Polyclinidae), and the larvae of *Didemnum* cf. *candidum* (Didemnidae), returned negative results.

Appendicularia

The reactivity with antibodies against serotonin in an oikopleurid appendicularian is seen in Fig. 4. Two cells in the posterior part of the neural ganglion are positively stained in *Oikopleura fusiformis* (double arrowhead and arrow in Fig. 4D, E). No other cellular structures stain in the nervous system of this species.

No labeling was detected in the central nervous system of *Oikopleura rufescens* (Oikopleuridae) and a single specimen of *Fritillaria* sp. (Fritillariidae).

Discussion

A morphologically distinct serotonergic nervous system with an anterior aggregation of cell bodies, a posterior network of fibers, and a posterior fiber projection is present in the phlebobranch and stolidobranch ascidian larvae and in the doliolid oozooid. A comparable serotonergic complex was not detected in appendicularians and aplousobranch ascidian larvae.

Homology of the serotonergic nervous systems

Several lines of evidence suggest homology of the serotonergic nervous systems present in Tunicata. (i) They are characterized by epitopes that are labeled by monoclonal antibodies against serotonin. (ii) All serotonergic components of nervous systems are situated in the anterior region of the dorsal central nervous system. (iii) Moreover, in phlebobranch and stolidobranch larvae, doliolid oozooids, and Notochordata, they are similar in organization and possibly in function. These similarities support the hypothesis that the observed serotonergic nervous systems are homologous.

Garstang (1928) suggested that Doliolida might be derived by neoteny from sessile ascidians. The similarity of the serotonergic cells in the dorsal ganglion of Doliolida to the serotonergic nervous system in larval stages could corroborate this hypothesis. Investigations of the nervous system of doliolid larvae will be of special interest.

Reduced serotonergic nervous system in aplousobranchiate larvae and Appendicularia

The lack of labeling with antibodies against serotonin in several species of aplousobranchiate ascidians and appendicularians could be the result of technical problems. For example, it is well known that penetration of antibodies into animals invested with cuticles can be problematic (e.g. Harlow and Lane 1998). However, in larvae of *Clavelina lepadiformis* and in *O. fusiformis*, cells in the dorsal nervous system were positively labeled. In *C. lepadiformis*, cells in the pharyngeal epithelium and the stomach were labeled as well. This indicates that the observed reduction of signal in the central nervous system was real. Nevertheless, it seems unlikely that the almost ubiquitous neurotransmitter serotonin would be reduced completely in the two clades. Serotonin could also be modified in these two groups. It is noteworthy in this context that serotonin was not detected by immunofluorescence in adult ascidians (Fritsch et al. 1982; Georges 1985), and that Appendicularia and Aplousobranchiata have been considered to show heterochrony. Thus, it is possible that a heterochronic modification of the serotonergic nervous system in the two groups is an evolutionarily derived shared character.

Reconstruction of the serotonergic nervous system of the last common ancestor of Tunicata and Chordata

The presence of a distinct serotonergic nervous system in phlebobranch and stolidobranch ascidian larvae and doliolid oozooids, but absence in Appendicularia and Aplousobranchiata, poses the question what character state was present in the last common ancestor of the Tunicata. Outgroup comparison can answer this question.

Fig. 5 depicts phylogenetic relationships between higher chordate taxa (Stach 2000; Nielsen 2001; Stach and Turbeville 2002). The relevant outgroup, the sister group of Tunicata, is Notochordata consisting of Cephalochordata + Craniata.

Both, the serotonergic nervous system in larval Cephalochordata (Holland and Holland 1993: p. 200, Fig. 4F, arrowhead) and Petromyzontida (Hay-Schmidt 2000: p. 1072, Fig. 1k), show an anterior aggregation of neurons with a posterior fiber network, and fibers projecting posteriorly. The same was reported from ontogenetic stages of *Xenopus laevis* (van Mier et al.

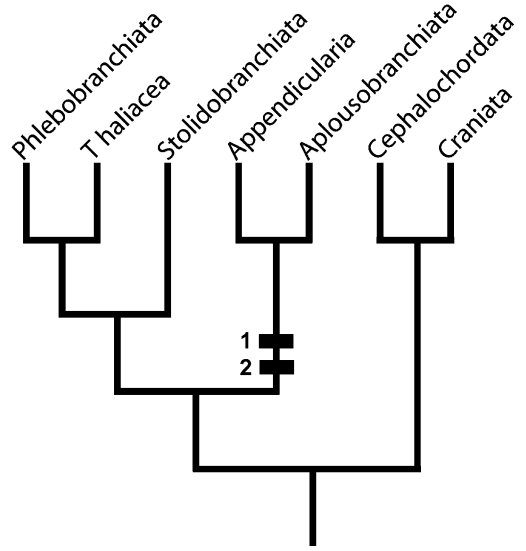

Fig. 5. Phylogenetic hypothesis for higher chordate taxa; relationships of higher tunicate taxa as suggested by a parsimony analysis of 18S rDNA sequences (Stach and Turbeville 2002); two indicated potential synapomorphies are: 1 = horizontal orientation of larval tail, 2 = reduction of serotonergic nervous system.

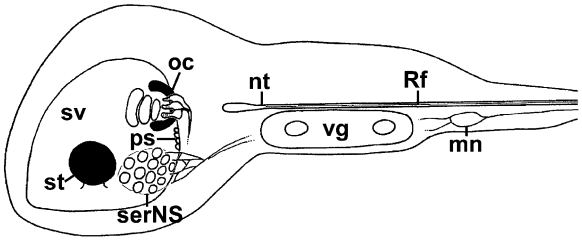

Fig. 6. Schematic representation of the tunicate larval central nervous system; based on Nicol and Meinertzhagen (1991); with selected morphological features added. Abbreviations: mn = motoneuron (after Okada et al. 2002), nt = neural tube, oc = ocellus (after Burighel and Cloney 1997), ps = pressure sensitive cells (after Nicol and Meinertzhagen 1991), Rf =Reissner's fiber (after Olsson 1993), serNS = serotonergic nervous system (present study), st = statocyte complex (after Dilly 1962), sv = sensory vesicle, vg = vegetal ganglion cells.

1986). Such a distinctly organized serotonergic nervous system therefore can be considered to be homologous and present in the last common ancestor of Notochordata, Tunicata, and Chordata.

The only study on the serotonergic nervous system of an ascidian larva found immunoreactivity against serotonin in the sensory vesicle, diffused in the ventral trunk region, in the papillae, and some cells in the tail of *P. mammillata* (Phlebobranchiata; Pennati et al. 2001). From the documentation published by these authors it is difficult to assess the morphology of the labeled cells and structures.

Comparison of the serotonergic nervous system to other deuterostomes

Cladistic analyses of morphological characters suggest that Enteropneusta might constitute the sister group to Chordata (Hay-Schmidt 2000; Nielsen 2001), whereas many molecular studies propose that a taxon comprising Hemichordata plus Echinodermata is the sister taxon to Chordata (recently reviewed; e.g., by Jenner and Schram 1999; Giribet 2002). Hay-Schmidt (2000) suggested that two major types of larval serotonergic nervous system could be distinguished in bilaterians. The first type consists of a fixed low number (around 5) of serotonergic cells and is found in protostomian taxa. The second type features numerous (>15) serotonergic neurons in the apical ganglion and posterior projecting axons, and is found in deuterostomes. The serotonergic nervous system in the groundplan of the Tunicata and Chordata reconstructed in the previous paragraphs clearly belongs to the deuterostome-like serotonergic nervous systems. Nezlin and Yushin (2004) demonstrated that serotonergic cells in tornaria larvae are concentrated in the apical ganglion, whereas in echinoderm larvae they are scattered along the ciliary band. Thus, the serotonergic nervous system in Enteropneusta seems more similar to that in the groundplan of Chordata.

Functional considerations

Fig. 6 shows a schematic indicating the position of the serotonergic nervous system as deduced from the present study. Position and cell numbers of the anterior aggregate of serotonergic cell bodies are similar to the cells called ND (dorsal neurons) and NV (ventral neurons) in the posterior sensory vesicle of *C. intestinalis* (Nicol and Meinertzhagen 1991). The structure named PSV by Takamura (1998) is also similar to the serotonergic group of cells.

The larval stage of sessile ascidians reacts to various environmental stimuli and plays a crucial role in dispersal and substrate selection (Svane and Young 1989; Vazquez and Young 1996; Meinertzhagen and Okamura 2001). In addition, McHenry and Strother have recently demonstrated that the locomotion of aplousobranchiate larvae is rather complicated and flexible (McHenry 2001; McHenry and Strother 2002).

The position of the serotonergic cell bodies close to the statocyte complex, and the posterior network, point to an integrative function for sensory input. Signal output could follow the posteriormost projection, which is directed toward the visceral ganglion, where individual motor neurons were characterized by Okada et al. (2002). The serotonergic neurons might therefore function in the coordination of locomotion, as is known for

a variety of taxa, such as molluscs (Norekian and Satterlie 2001; Yu et al. 2001) or hirudinean annelids (Nusbaum and Kristan 1986). Serotonin also modulates the locomotor behavior in lampreys (Grillner and Matsushima 1991), and serotonergic neurons project towards motoneurons during *X. laevis* ontogeny (van Mier et al. 1986). However, serotonin serves a wide range of functions as a signal molecule during ontogeny. It modulates ciliary activity, regulates metamorphosis, and in craniates functions in modulation of pain reception, regulation of sleep-waking cycles, autonomic functions and reproductive behavior.

Based on the association of serotonergic cells with the sensory vesicle, Pennati et al. (2001) suggested that serotonergic cells were involved in light detection in the larva of *P. mammillata* (Phlebobranchiata). The higher resolution as well as better fixation and labeling achieved in the present study demonstrate that the serotonergic perikarya are actually closely associated with the statocyte complex. However, the possibility that the serotonergic nervous system receives inputs from the light-sensitive ocellus cannot be excluded. In fact, Takamura (1998) revealed fibers emerging from the ocellus directed towards the region of the central nervous system where the serotonergic neurons were discovered in the present study. Electrophysiological experiments will be necessary to decide between the two hypotheses.

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